American Journal of Pathology, Vol. 152, No. 6, June 1998

Endothelial Gaps and Adherent Leukocytes in Allergen-Induced Early- and Late-Phase Plasma Leakage in Rat Airways

Peter Baluk, Patrick Bolton, Akira Hirata, Gavin Thurston, and Donald M. McDonald

From the Cardiovascular Research Institute and Department of Anatomy, University of California, San Francisco, San Francisco, California

Exposure of sensitized individuals to antigen can induce allergic responses in the respiratory tract, manifested by early and late phases of vasodilatation, plasma leakage, leukocyte influx, and bronchoconstriction. Similar responses can occur in the skin, eye, and gastrointestinal tract. The early-phase response involves mast cell mediators and the late-phase response is leukocyte dependent, but the mechanism of leakage is not understood. We sought to identify the leaky blood vessels, to determine whether these vessels contained endothelial gaps, and to analyze the relationship of the gaps to adherent leukocytes, using biotinylated lectins or silver nitrate to stain the cells *in situ* and Monastral blue as a tracer to quantify plasma leakage. Most of the leakage occurred in postcapillary venules (<40-μm diameter), whereas most of the leukocyte migration (predominantly neutrophils) occurred in collecting venules. Capillaries and arterioles did not leak. Endothelial gaps were found in the leaky venules, both by silver nitrate staining and by scanning electron microscopy, and 94% of the gaps were distinct from sites of leukocyte adhesion or migration. We conclude that endothelial gaps contribute to both early and late phases of plasma leakage induced by antigen, but most leakage occurs upstream to sites of leukocyte adhesion. *(Am J Pathol 1998, 152:1463–1476)*

Inhalation of allergen by sensitized individuals can trigger a biphasic inflammatory response in the airways, with an early transient response within a few minutes of the exposure and a prolonged late-phase response starting 3 to 8 hours later.[1,2] Corresponding allergic responses can occur in the nose, skin, eye, and intestinal tract.[2]

Many studies have focused on changes in airflow resistance and the influx of leukocytes into the airways.[3–5] However, relatively few studies have examined the contribution of microvascular leakage to the inflammatory process, although extravasation of plasma can result in airway edema and narrowing.[6,7] The early phase of plasma leakage seems to involve the activation of mast cells and release of diffusible inflammatory mediators. These mediators in turn are believed to recruit leukocytes, on which the late-phase plasma leakage may be dependent.[2,8]

Plasma leakage produced by rapidly acting inflammatory mediators such as histamine, serotonin, bradykinin, or substance P results from intercellular gaps that form in the endothelium of postcapillary venules.[9,10] However, in some pathological conditions where prolonged leakage occurs, eg, after certain bacterial toxins or mild thermal or ultraviolet light injury, arterioles and capillaries may be involved along with venules.[11]

The location and mechanism of plasma leakage associated with early or late phase plasma leakage are unknown. One possibility is that the leakage occurs through endothelial gaps in postcapillary venules, as in the leakage induced by histamine-type mediators. However, the dependence of the late-phase plasma leakage on the influx of leukocytes raises the possibility that the leakage occurs at the sites where leukocytes migrate through the endothelium. Alternatively, leakage could occur through transendothelial holes, variously termed transcellular openings, channels, or pores,[12] by way of vesiculo-vacuolar organelles[13] or by the transcytosis of vesicles or caveolae.[14]

This study had two specific aims. First, we sought to identify the leaky vessels and determine whether endothelial gaps contribute to early- or late-phase plasma leakage. Second, we sought to determine the relationship of endothelial gaps to adherent or migrating leukocytes.

The model we used stemmed from our previous experiments on Brown Norway rats.[15] First, by comparing responses in Brown Norway, Lewis, F344, and Wistar rats, we identified the strain that has the largest late-phase

Supported in part by grants from the National Institutes of Health (HL-24136), Glaxo-Wellcome Inc., Research Triangle Park, NC, the Research Evaluation and Allocation Committee, and the Academic Senate Committee on Research at the University of California, San Francisco.

Accepted for publication March 17, 1998.

Dr. Hirata's present address is Departments of Anatomy and Opthalmology, Kumamoto University School of Medicine, Kumamoto 860, Japan.

Address reprint requests to Dr. Peter Baluk, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0130. E-mail: pbaluk@itsa.ucsf.edu.

leakage in the respiratory tract. Next, using this strain (Wistar rats), we measured the amount and time course of leakage with Evans blue.[15] Individual leaky blood vessels were identified histologically in tracheal whole mounts using Monastral blue as a tracer, in combination with endothelial staining with a biotinylated lectin or silver nitrate.[10,16] These specimens were also used to localize and quantify adherent or migrating leukocytes. Endothelial gaps and sites of leukocyte migration were identified by scanning electron microscopy (EM) and silver nitrate staining.[10,17,18]

Materials and Methods

Animals

Pathogen-free Wistar male rats (Charles River, Hollister, CA) were housed in microisolator units under barrier conditions. The rats were 7 to 8 weeks of age and 176 to 200 g in body weight on arrival. Brown Norway and Lewis rats (Charles River, Kingston, NY) and F344 rats (Simonsen Laboratories, Gilroy, CA) were also used in comparative studies (n = 4 to 6 rats per group). All experimental procedures were approved by the Committee on Animal Research at the University of California, San Francisco.

Antigen Sensitization and Challenge

One day after arrival, rats were anesthetized with ketamine (50 mg/kg intraperitoneally (i.p.)) and xylazine (2 mg/kg i.p.) and were injected subcutaneously with a suspension containing 1 mg of ovalbumin (grade V, Sigma Chemical Co., St. Louis, MO) and 200 mg of aluminum hydroxide (Aldrich, Milwaukee, WI) in sterile 0.9% NaCl.[15] At the same time, 1 ml of *Bordetella pertussis* vaccine (IAF Biovac, La Val, Quebec, Canada) containing 8×10^9 heat-killed bacilli was injected i.p. as an adjuvant. Unanesthetized rats were challenged 12 to 16 days after sensitization by nose-only exposure to an aerosol of ovalbumin for 30 minutes.[15] During the challenge, the rats were restrained in a wire net holder attached to an ultrasonic nebulizer (DeVilbiss model 5000D, Somerset, PA) containing 5 ml of ovalbumin solution (5 mg/ml in sterile 0.9% NaCl). The aerosol was propelled by a 250 ml/minute flow of type 1 grade breathing air. Control (baseline) rats were neither sensitized nor challenged.

Measurement of Plasma Leakage

In early-phase experiments, unanesthetized rats were given Evans blue (30 mg/kg intravenously (i.v.); EM Sciences, Cherry Hill, NJ) via a lateral tail vein and were then challenged with ovalbumin for 30 minutes. Immediately thereafter, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused with fixative, 1% paraformaldehyde in 0.05 mol/L citrate buffer, pH 3.5,[15] 40 minutes after the onset of the challenge.

In late-phase experiments, unanesthetized rats were challenged with ovalbumin, as above, and then allowed to wait for various intervals before anesthesia. Evans blue was injected i.v., and 30 minutes later the rats were perfused with fixative. The perfusion occurred 1, 2, 4, 6, 8, or 24 hours after the end of the challenge.[15] After the perfusion, the tracheas were dissected, blotted, and weighed. Evans blue was extracted, measured with a spectrophotometer, and expressed as nanograms of dye per milligram of tracheal tissue.[15]

Identification of Leaky Vessels

Monastral blue was injected 10 minutes before fixation to mark sites of plasma leakage.[18] Rats were challenged and, 10 minutes later or at the peak of late-phase leakage 4 hours later, were perfused for 5 minutes with fixative (1% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline, pH 7.4). After fixation, tracheal blood vessels were stained with biotinylated concanavalin A lectin (Vector Laboratories, Burlingame, CA) and visualized using a peroxidase-diaminobenzidine reaction.[16] Tracheas were dehydrated in ethanol, cleared in toluene, and prepared as flat whole mounts.

The area density of extravasated Monastral blue was measured in vessel walls in five to six tracheas per group. Live video images of lectin-stained vessels from a microscope (Zeiss Axiophot) equipped with a 40× NA 1.0 objective lens and a color video camera (Sony 3 CCD model DXC 755) were projected on a video monitor. The number of pixels within an 80 × 80 pixel square (corresponding to 20 × 20 μm region of vessel wall) that fell within the color threshold of Monastral blue was determined using a Videometric 150 imaging system (Oncor, Gaithersburg, MD). Threshold values of hue, luminosity, and saturation were set to include only Monastral blue. The amount of Monastral blue was expressed as the percentage of pixels that fell within the threshold for Monastral blue. The diameter of each vessel was also measured. In each trachea, the amount of extravasated Monastral blue was assessed in 10 mucosal vessels in each of six categories (arterioles, capillaries, postcapillary venules (<20 μm or 20 to 40 μm), and collecting venules (40 to 60 μm or >60 μm in diameter)).

Identification and Quantification of Endothelial Gaps

Scanning Electron Microscopy

The ultrastructure of the endothelial surface of tracheal blood vessels was examined by scanning EM. At 10 minutes or 4 hours after antigen challenge, the vasculature was perfused with fixative (1% paraformaldehyde and 0.5% glutaraldehyde in 0.075 mol/L cacodylate buffer, pH 7.4) for 5 minutes.[17,18] Tracheas were embedded in agarose and cut with a Vibratome into 100-μm-thick cross sections to expose the lumen of vessels. The sections were prepared for scanning EM and were observed with a JSM 840A scanning electron microscope.[17,18]

Silver Nitrate Staining

At 10 minutes or 4 hours after antigen challenge, the vasculature was perfused with fixative (1% paraformaldehyde and 0.5% glutaraldehyde in 0.075 mol/L cacodylate buffer, pH 7.4) for 5 minutes. Monastral blue was injected 10 minutes before fixation to mark sites of plasma leakage. After fixation, the endothelial cells were stained with silver nitrate, and tracheas were prepared as flat whole mounts.[10] The number of endothelial gaps was inferred from the number of focal, dot-like silver deposits along the silver lines at endothelial borders after silver nitrate staining.[10] The number of silver dots per cell was determined for 25 silver-stained endothelial cells each in postcapillary venules (<40 μm in diameter) and in collecting venules (>40 μm in diameter) in six tracheas per group at a projected magnification of ×3700. The diameter of 25 silver dots was measured in each of four late-phase tracheas at a projected magnification of ×7100. The size and shape of 100 consecutive endothelial gaps in early and in late phase specimens was also assessed in scanning EM micrographs at magnifications of ×2000 to ×10,000.[17]

Identification and Quantification of Adherent Intravascular Leukocytes

Adherent leukocytes were identified and counted in lectin-stained blood vessels. Neutrophils were identified by their golden-brown peroxidase-positive cytoplasmic granules, eosinophils by their large size and distinctive dark brown peroxidase-positive granules, monocytes by their large size, ameboid shape, and absence of granular staining, and lymphocytes by their small size, spherical shape, and absence of granular staining.[15] Leukocyte densities were expressed as number of cells per square millimeter of mucosal surface or per square millimeter of endothelial surface.

Migrating leukocytes, which appear as silver rings at endothelial cell borders,[10] were counted for 25 silver-stained endothelial cells each in postcapillary venules (<40 μm in diameter) and in collecting venules (>40 μm in diameter) in six tracheas per group at a projected magnification of ×3700. The number of silver rings coincident with silver dots (endothelial gaps) was also recorded. The diameter of 25 silver rings and 25 leukocytes was measured in each of four silver-nitrate-stained late-phase tracheas at a projected magnification of ×7100. The relationship between leukocytes and endothelium was also assessed by scanning EM at magnifications of ×2000 to ×10,000.

Statistical Analysis

Values are presented as means ± SE (n = 6 unless otherwise indicated). The significance of differences between groups was determined by analysis of variance followed by Scheffé's F-test for multiple comparisons. Population distributions of plasma leakage, endothelial gaps, and migrating leukocytes in venules of different size were compared by the Kolmogorov-Smirnov two-sample test. Differences were considered significant when $P < 0.05$.

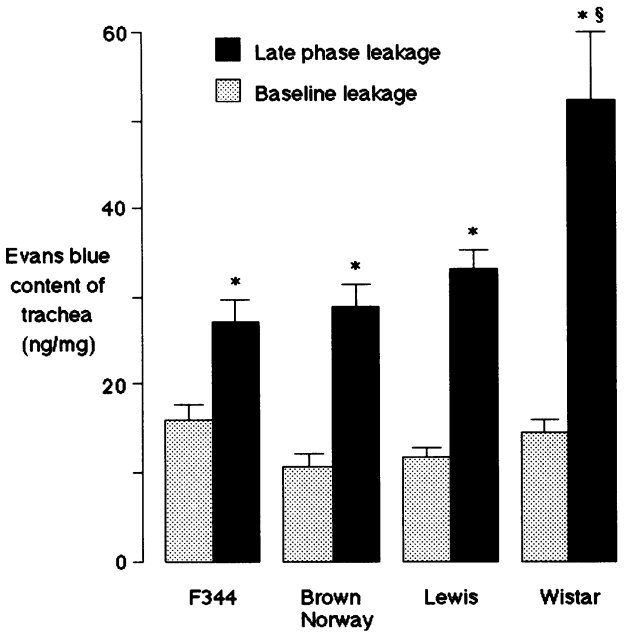


Figure 1. Comparison of amounts of late-phase plasma leakage (4 hours after ovalbumin challenge) in tracheas of sensitized rats. Baseline values were measured in unsensitized, unchallenged rats. Values are means ± SE; n = 6 rats per group. *$P < 0.05$ compared with baseline group; §$P < 0.05$ compared with other late-phase groups.

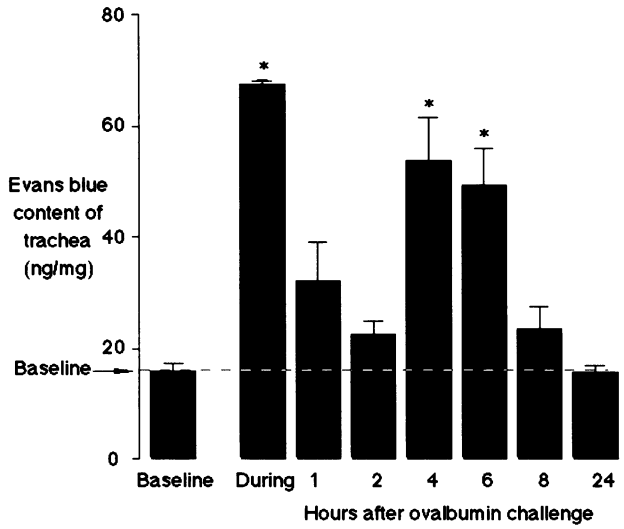
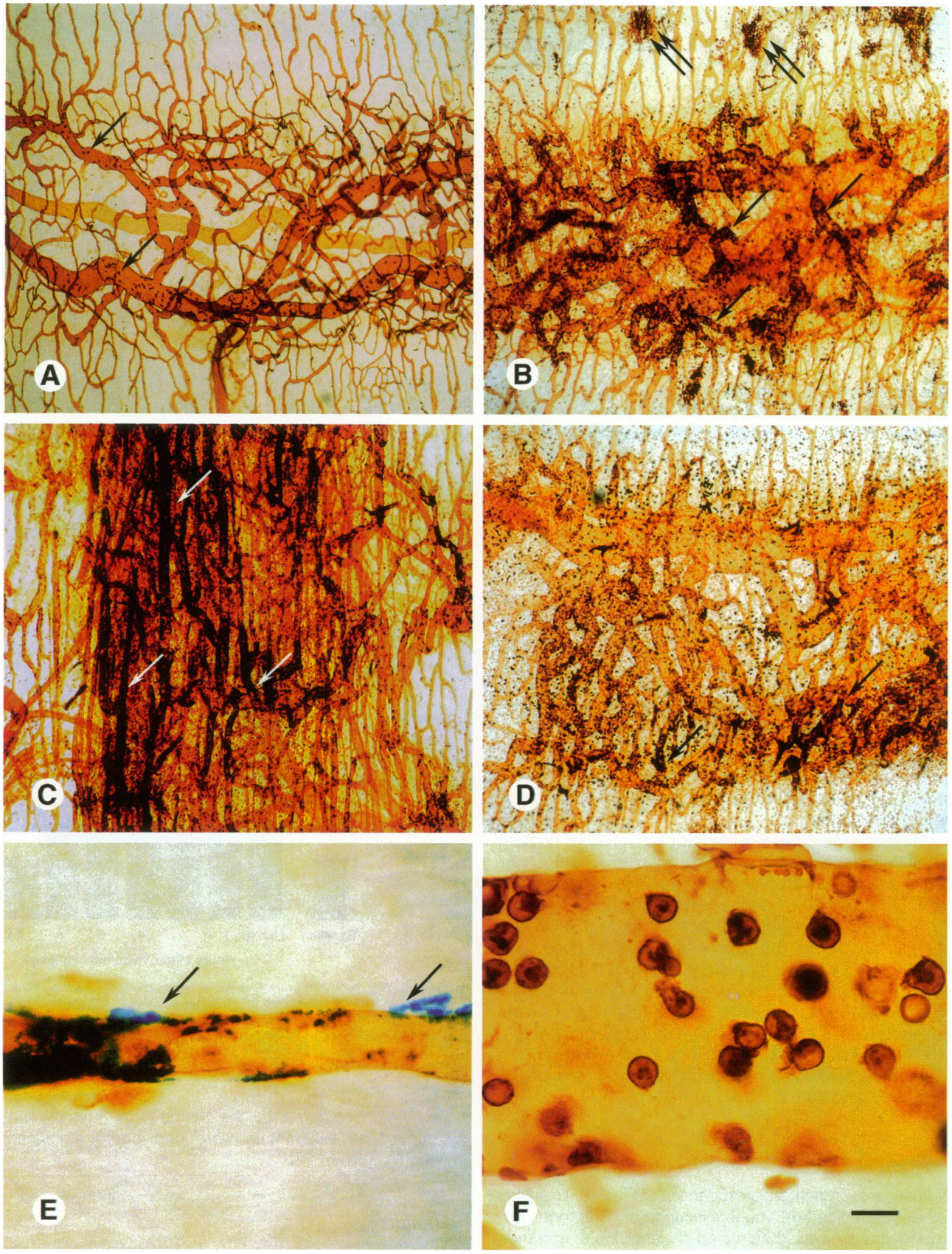


Figure 2. Time course of Evans blue leakage in tracheas of sensitized Wistar rats challenged with ovalbumin for 30 minutes. Baseline values were measured in unsensitized, unchallenged rats. Leakage during the challenge was measured by injecting Evans blue before the challenge. Values are means ± SE; n = 4 to 6 rats per group. *$P < 0.05$ compared with baseline group.

Results

Baseline plasma leakage was approximately the same in Wistar, Brown Norway, Lewis, and F344 rats, but the leakage 4 hours after ovalbumin challenge was nearly


A
B
C
D
E
F

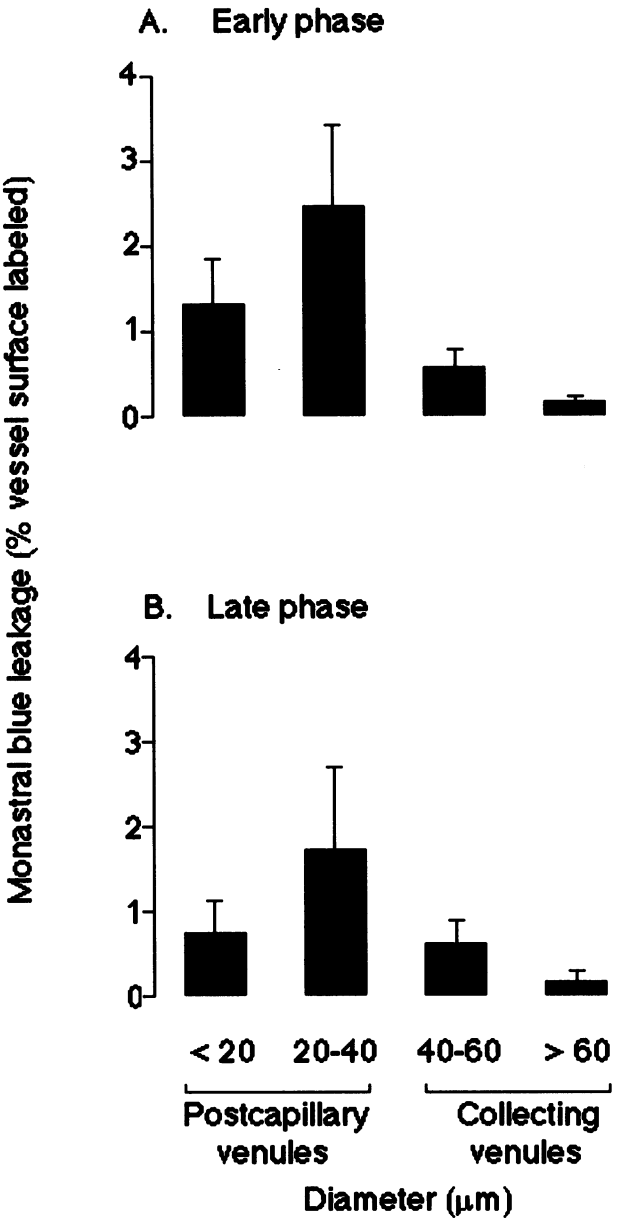


Figure 4. Comparison of amounts of early- and late-phase leakage of Monastral blue from venules of different sizes after ovalbumin challenge in tracheal mucosa between cartilage rings in sensitized Wistar rats. Values are means ± SE; n = 4 to 6 rats per group.

twice as much in Wistar rats as in the other strains (Figure 1). Based on these results, we used Wistar rats for all additional studies.

Time Course of Plasma Leakage after Ovalbumin Challenge

There were two peaks in Evans blue leakage after ovalbumin challenge (Figure 2). The first peak occurred during the challenge and was designated the early phase. This peak (67.4 ± 0.7 ng/mg) was approximately 4 times the baseline leakage in unsensitized, unchallenged Wistar rats (15.8 ± 1.4 ng/mg). The leakage was less at 1 hour and close to baseline at 2 hours after the challenge (Figure 2). The second peak occurred 4 hours after challenge. The leakage at 4 hours (53.7 ± 7.7 ng/mg) was approximately 80% of the early-phase peak, but it was part of a more prolonged phase of leakage, which was still present at 6 hours. There was less leakage at 8 hours and none above baseline at 24 hours (Figure 2). Leakage was not significantly different from baseline in rats that were challenged but not sensitized (14.5 ± 1.3 ng/mg at 4 hours) or were sensitized but not challenged (16.3 ± 2.2 ng/mg).

Identification of Leaky Vessels after Ovalbumin Challenge

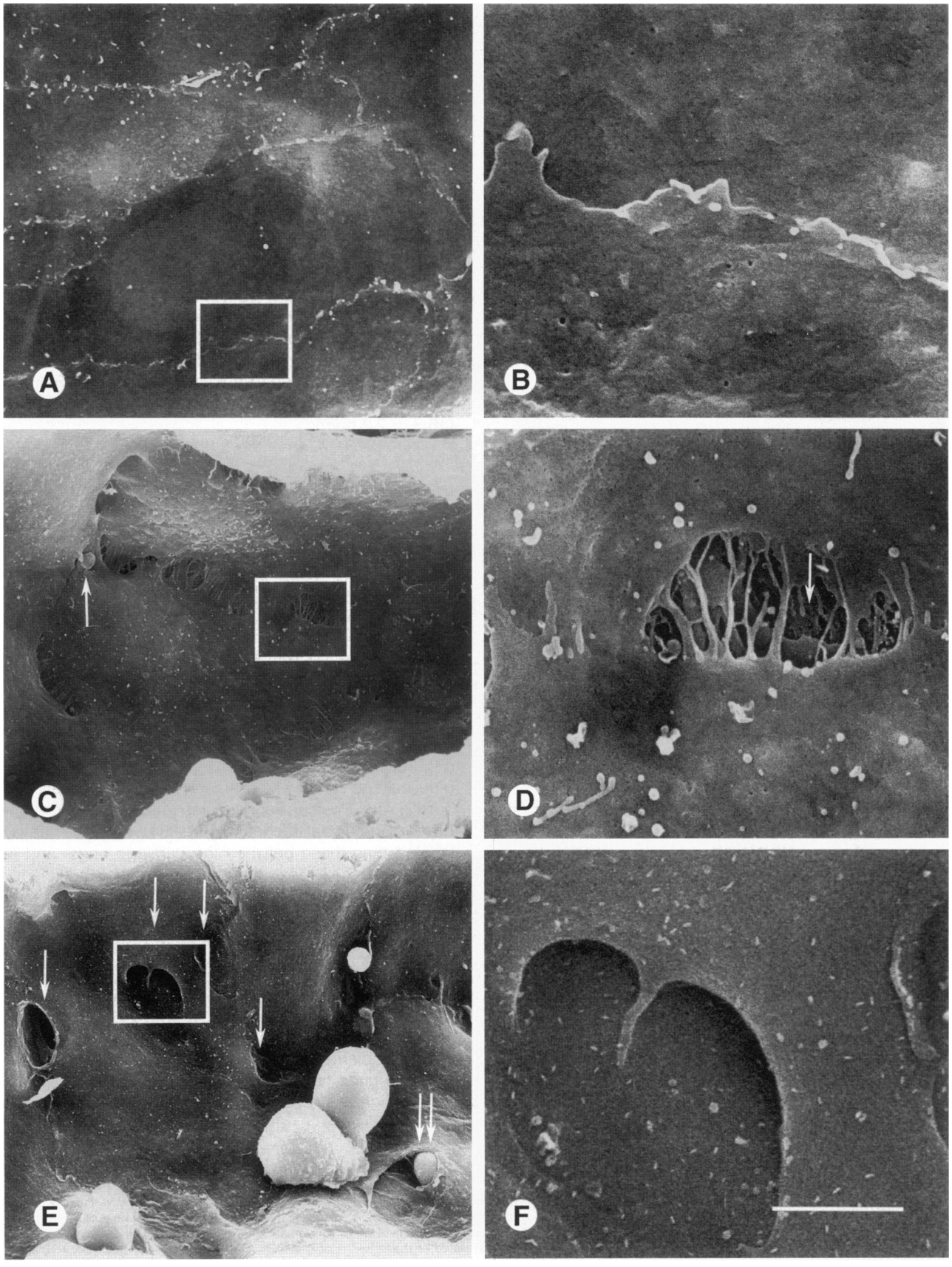
Baseline and Early-Phase Leakage

Under baseline conditions, no extravasated Monastral blue was detected in the tracheal vasculature, as visualized by lectin staining (Figure 3A). However, at 10 minutes after the challenge, the walls of some mucosal venules between the cartilage rings had scattered regions of extravasated Monastral blue (Figure 3B). Postcapillary venules 20 to 40 μm in diameter had the most labeling; collecting venules of all sizes had much less (Figures 3, B and C, and 4A). The amount of Monastral blue leakage varied widely among individual vessels and among rats. Postcapillary venules in the posterior membrane of the rostral trachea (Figure 3C) were more heavily labeled than those between the cartilage rings, and in some regions the extravasated tracer was sufficiently abundant to be visible to the naked eye. No Monastral blue labeling was found in the walls of arterioles or capillaries in either region of the trachea.

Late-Phase Leakage

At 4 hours after challenge, postcapillary venules 20 to 40 μm in diameter located in the mucosa between the cartilage rings had the most intense Monastral blue labeling (Figures 3, D and E, and 4B). Larger venules were

Figure 3. Monastral blue leakage in tracheas of control and ovalbumin-sensitized and challenged Wistar rats. Blood vessels were stained by vascular perfusion of biotinylated concanavalin A lectin and viewed from the luminal surface of tracheal whole mounts. **A:** Vessels in mucosa between cartilage rings of unsensitized, unchallenged rat. No leakage of Monastral blue and few intravascular leukocytes (**arrows**) are evident. **B:** Early-phase leakage of Monastral blue from postcapillary venules (**arrows**) in mucosa between cartilage rings. Scattered clusters of extravasated erythrocytes are also evident (**double arrows**). **C:** Extensive leakage of Monastral blue from postcapillary venules (**arrows**) in mucosa of posterior membrane during the challenge (early phase). **D:** Scattered regions of late phase (4 hours) leakage of Monastral blue from postcapillary venules (**arrows**) in mucosa between cartilage rings. Intravascular and extravascular leukocytes are abundant. **E:** Late-phase (4 hours) leakage of Monastral blue (**arrows**) from small postcapillary venule. No adherent leukocytes are present. **F:** Many adherent leukocytes, predominantly neutrophils, but no extravasated Monastral blue in collecting venule at 4 hours. Scale bar, 80 μm (**A** to **D**) and 10 μm (**E** and **F**).


A
B
C
D
E
F

sparsely labeled (Figures 3F and 4B). The average amount of Monastral blue labeling was approximately the same as in the early phase (Figure 4B), but venules in the mucosa over the posterior membrane had much less labeling than in the early phase.

Identification and Quantification of Endothelial Gaps

Baseline

When viewed by scanning EM, most endothelial cells of venules in unsensitized, unchallenged rats had a smooth surface. The endothelial borders were unruffled, junctions between cells appeared to be tightly apposed, and no endothelial gaps were observed (Figure 5, A and B). Consistent with these findings, the borders of endothelial cells stained with silver nitrate were outlined by continuous black lines (Figure 6A), and the number of silver dots at cell borders was practically zero (0.03 ± 0.02 silver dots per endothelial cell for all venules).

Early-Phase Leakage

At 10 minutes after challenge, endothelial cell borders of venules examined by scanning EM were ruffled and irregular and had focal intercellular gaps (Figure 5C). These gaps, termed vertical gaps, were similar to those seen 1 minute after exposure to substance P.[17] Most of these gaps exposed the basement membrane or an underlying cell (Figure 5, C and D). The gaps were roughly oval (major axis, 0.76 ± 0.03 μm; n = 85 gaps) and usually were oriented perpendicular to the endothelial cell border. Some of the larger openings were partitioned into multiple gaps by finger-like cytoplasmic processes up to 2.5 μm long (Figure 5D). Other endothelial cell junctions were separated by crescent-shaped, obliquely oriented slits between the overlapping endothelial cell borders (Figure 5, E and F). The basement membrane was not visible through these gaps. Such gaps, termed oblique slits,[17] averaged 1.27 ± 0.24 μm in length (n = 15 slits). The width of the separation at slits could not be measured reliably (Figure 5, E and F). Eighty-five percent of the gaps were of the vertical type and fifteen percent were oblique slits (n = 100 gaps). No gaps were seen in the endothelium of arterioles, and no transcellular holes were found in the endothelium of any vessels.

After silver nitrate staining, silver dots (1.2 ± 0.06 μm in diameter) were visible at the borders of some endothelial cells in regions of Monastral blue labeling (Figure 6B). Significantly more dots were found in postcapillary venules (20 to 40 μm in diameter) in the posterior membrane than in the corresponding venules between the cartilage rings (7.3 ± 0.9 *versus* 3.7 ± 0.2 silver dots per endothelial cell).

Late-Phase Leakage

In venules examined by scanning EM 4 hours after challenge, endothelial cell borders were more ruffled than in baseline controls, but they were less ruffled than in the early phase (Figure 7, A and B). Also, endothelial gaps were less numerous than in the early phase. Only 30% were vertical gaps; the remainder (70%) were oblique slits (Figure 7, B–E; n = 100 gaps). The average sizes of vertical gaps (0.64 ± 0.06 μm) and oblique slits (1.36 ± 0.11 μm) were not significantly different from corresponding values for the early phase. None of the large gaps were partitioned by finger-like processes.

In late-phase specimens stained with silver nitrate, as in the early phase, silver dots were most numerous in postcapillary venules 20 to 40 μm in diameter (Figures 6C and 8B). Silver dots were equally common in the early and late phase (3.7 ± 0.2 *versus* 3.8 ± 0.3 silver dots per endothelial cell, respectively, in venules located between the cartilage rings).

Disassociation of Adherent Intravascular Leukocytes and Sites of Leakage

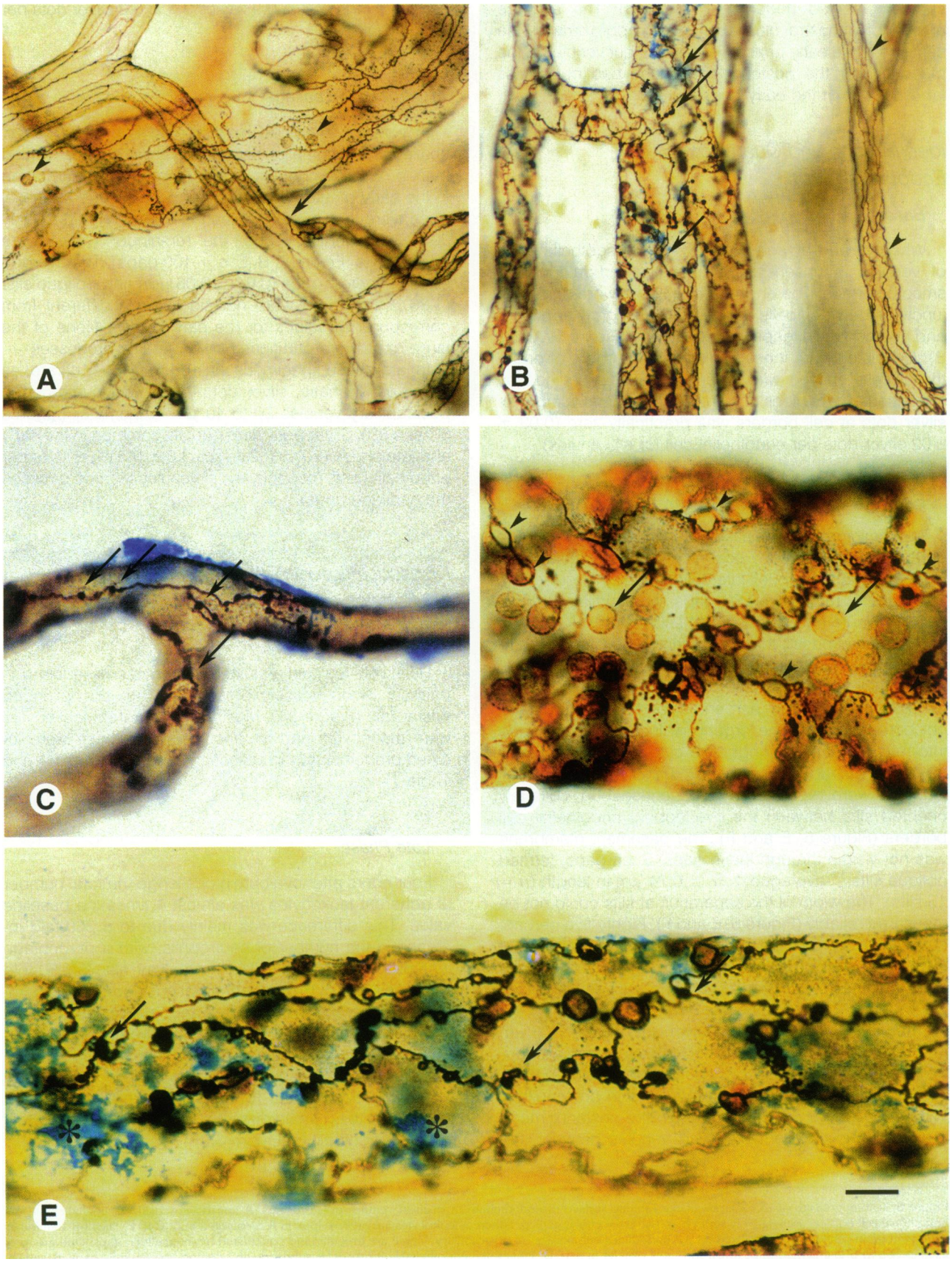
Baseline

Relatively few lectin-stained leukocytes were found in tracheal blood vessels of unsensitized, unchallenged Wistar rats (Figure 3A). Most of the adherent leukocytes were monocytes and lymphocytes, and these were located predominantly in collecting venules (Figure 3A and Table 1).

Late Phase

Four hours after ovalbumin challenge the total number of adherent leukocytes was almost 4 times the baseline value. This difference was mainly due to a 200-fold increase in the number of neutrophils, with a percentage increase from 1% to 57%. There was also an 80-fold increase in the number of adherent eosinophils. The number of adherent lymphocytes and monocytes did not increase significantly (Table 1). Adherent leukocytes were scattered in postcapillary venules <20 μm in diameter (170 ± 41 leukocytes/mm^2 of endothelium; Figure 3E) but were more than 10-fold as numerous in collecting venules 40 to 60 μm in diameter (1920 ± 197 leukocytes/mm^2 of endothelium; Figure 3F).

Figure 5. Scanning electron micrographs of the luminal surface of endothelium in tracheal venules of Wistar rats. **A** and **B**: Unsensitized, unchallenged rat. Endothelial cell borders are relatively smooth and no intercellular gaps or adherent leukocytes are present. Box in **A** is enlarged in **B**. **C** to **F**: Fixation 10 minutes after ovalbumin challenge of sensitized rat. **C**: Endothelial cell borders in postcapillary venule are irregular and punctuated by intercellular gaps. One group of gaps in box in **C** is enlarged in **D**; **arrow** indicates platelet in gap. **D**: Finger-like endothelial cell processes subdivide an opening into multiple gaps. Basement membrane (**arrow**) is visible through gap. **E**: Endothelium of collecting venule showing multiple oblique slit-like gaps (**arrows**) at endothelial cell margins. Several leukocytes adhere to the endothelium, and one is migrating (**double arrows**). **F**: Enlargement of oblique slit-like gaps in box in **E**. Scale bar, 10 μm (**A**, **C**, and **E**) and 2 μm (**B**, **D**, and **F**).


A
B
C
D
E

The distribution of adherent lectin-stained leukocytes was significantly different from the distribution of extravasated Monastral blue in venules of different diameter (Kolmogorov-Smirnov two-sample test, $P < 0.05$). In addition to the adherent intravascular leukocytes, many extravascular leukocytes were scattered in the mucosa (Figure 3D).

Relationship of Endothelial Gaps to Sites of Leukocyte Migration

Baseline and Early Phase

More adherent cells were found by scanning EM and in silver-nitrate-stained preparations at 10 minutes after ovalbumin challenge than under baseline conditions (Figure 5E). In addition to the spherical cells scattered over the endothelium, numerous elongated or tear-shaped leukocytes protruded through the endothelium at sites marked by silver rings (1.2 ± 0.2 per endothelial cell; n = 6 rats), representing putative sites of leukocyte migration (Figure 6, D and E). Such silver rings were rare under baseline conditions (0.08 ± 0.02 silver rings per endothelial cell). Silver rings (diameter, 3.7 ± 0.3 μm; n = 4 rats), which were significantly smaller than the leukocytes (diameter, 6.3 ± 0.1 μm; n = 4 rats), were most numerous on collecting venules. Postcapillary venules <20 μm in diameter had few silver rings (Figure 9A). Few endothelial gaps marked by silver dots were associated with leukocytes; only 6% of silver dots were located on silver rings, and only 11% of the rings had silver dots.

Late Phase

Adherent leukocytes were abundant in vessels 4 hours after ovalbumin challenge (Figure 7A). Sometimes, the position of a leukocyte that had already migrated could be detected as a bulge in the overlying endothelium (Figure 7B). Most endothelial gaps examined by scanning EM were not associated with adherent or migrating leukocytes (Figure 7, C–E). Conversely, in most cases (27 of 36), no gaps were found next to migrating leukocytes.

Migrating leukocytes identified by silver rings at endothelial cell borders were approximately as numerous in the early phase as in the late phase (1.2 ± 0.2 *versus* 1.6 ± 0.1 silver rings per endothelial cell, $P = 0.4$; Figure 6, D and E). As in the early phase, silver rings were located preferentially on the larger venules (>40 μm). The smallest postcapillary venules had few silver rings, none of which had silver dots (Figures 6C and 9B). Also, there was no apparent relationship between the amount of plasma leakage and the number of migrating leukocytes in particular vessels, because extravasated Monastral blue and the number of silver rings had significantly different distributions in venules of different sizes (Figures 4B and 9B; Kolmogorov-Smirnov two-sample test, $P < 0.05$). Small amounts of extravasated Monastral blue were found at or near some silver rings with silver dots but not at rings without silver dots (Figure 6E).

Discussion

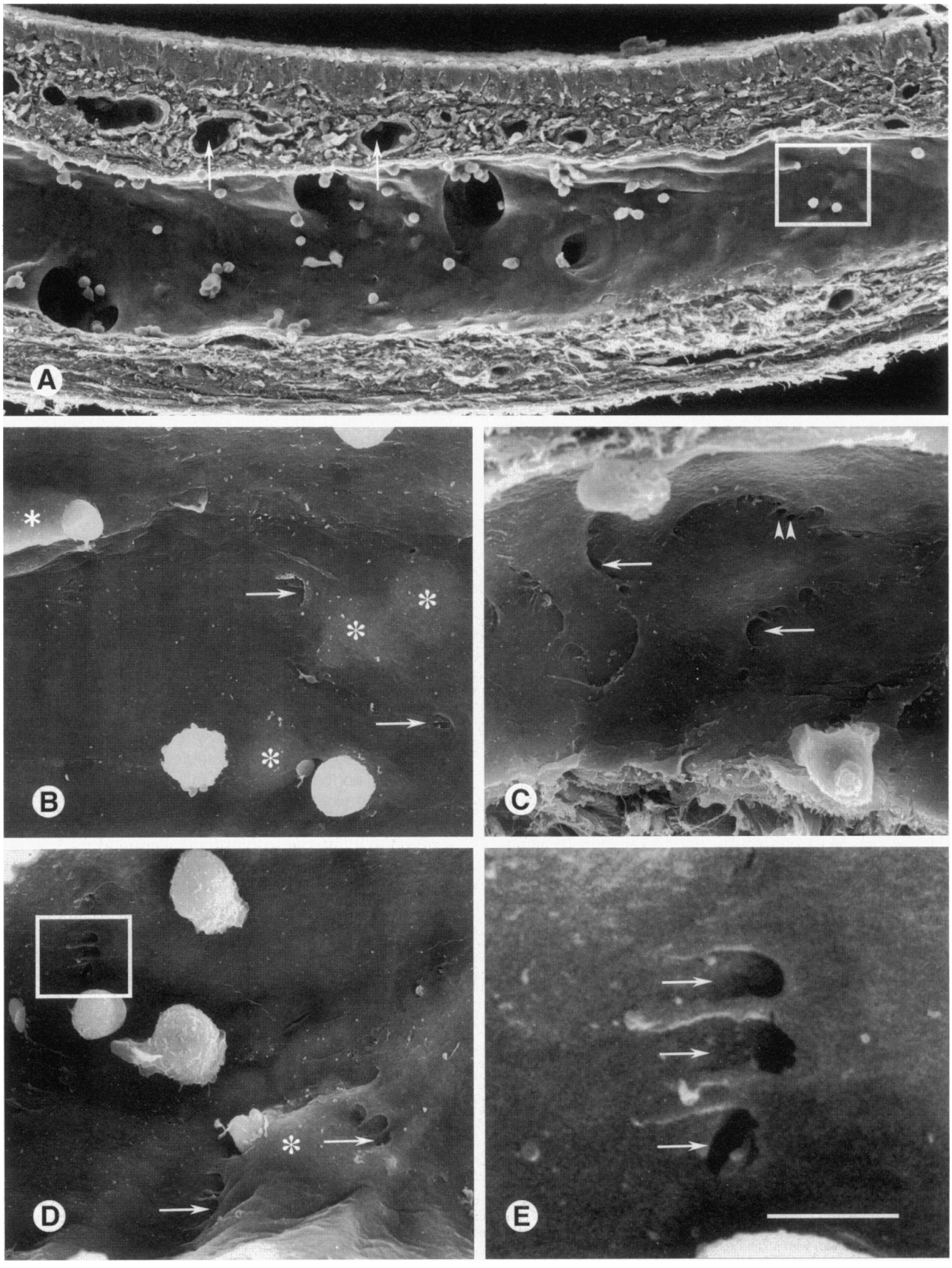
The present study addressed the time course, location, and mechanism of antigen-induced plasma leakage in rat airways. We found that antigen challenge resulted in early and late phases of plasma leakage. The late-phase leakage was much greater in Wistar rats than in Brown-Norway, Lewis, or F344 rats. In both phases, the leakage was greatest in postcapillary venules that contained numerous endothelial gaps. Adherent and migrating leukocytes were also abundant in the microvasculature, but they were most numerous in collecting venules where little leakage occurred. Thus, most of the leakage occurred upstream to the sites of leukocyte migration.

Features of the Model

We were surprised to find that antigen exposure induced much more late-phase plasma leakage in the airways of sensitized Wistar rats than in Brown Norway, Lewis, or F344 rats. Early-phase leakage and late-phase leukocyte influx was also greater in Wistar rats than in our earlier study in Brown Norway rats. Despite the differences in magnitude, the time course of allergen-induced leakage in Wistar rats was similar to what we found earlier in Brown Norway rats.[15] Most studies of late changes in airflow resistance and cellular influx in rat airways have used Brown Norway rats because of their large IgE response.[19–21] Our findings on leakage are consistent with a report that late-phase plasma leakage induced by repeated challenges with *Ascaris* antigen is greater in Wistar rats than in Brown Norway rats.[22] A recent study of late-phase leukocyte influx into the lungs of sensitized rats reported large effects in ovalbumin-sensitized and -challenged Brown Norway rats, but essentially no inflammation was detected in F344 or Lewis rats[5]; Wistar rats were not studied.

The sensitization procedure that we adopted in this and a previous study[15] was used because it promotes the occurrence of late-phase reactions in rats, and it has been widely used in studies of late-phase bronchoconstriction and leukocyte influx.[4,5] The composition of the sensitization mixture and the route of administration may

Figure 6. Whole mounts of tracheas of Wistar rats. Blood vessels were stained by perfusion of silver nitrate, and leakage was detected by extravasation of Monastral blue. **A:** Baseline conditions. No silver dots and no leakage of Monastral blue is seen. Arteriole is indicated by an **arrow**. Also seen is a venule containing adherent leukocytes (**arrowheads**). **B:** Early-phase leakage (during the challenge) in postcapillary venule with endothelial gaps marked by silver dots (**arrows**). Arteriole (**arrowheads**) with no leakage or silver dots is seen. **C:** Late-phase leakage (4 hours) of Monastral blue near silver dots (**arrows**) on a small venule. No adherent leukocytes are seen. **D:** Many adherent leukocytes, visible as golden spherical cells (**arrows**), and several migrating leukocytes, visible as tear-shaped cells protruding through silver rings (**arrowheads**), in collecting venule 4 hours after challenge. No extravasated Monastral blue is visible. **E:** Collecting venule with small amount of Monastral blue leakage 4 hours after ovalbumin challenge. Most of the Monastral blue is near silver dots (endothelial gaps), not near silver rings (migrating leukocytes). **Arrows** mark examples of silver dots associated with silver rings. Scale bar, 50 μm (**A** and **B**), 25 μm (**C** and **D**), and 20 μm (**E**).


A
B
C
D
E

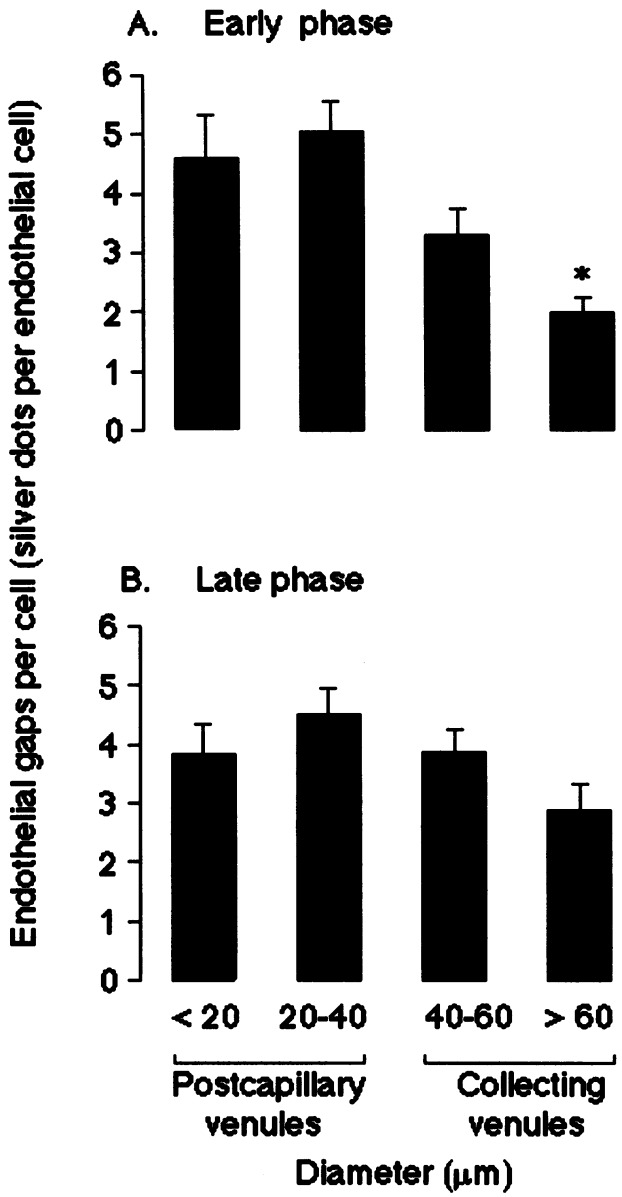


Figure 8. Number of endothelial gaps, visualized and quantified as silver dots after silver nitrate staining, during the early and late phases of plasma leakage after ovalbumin challenge in tracheal mucosa (between cartilage rings) in sensitized Wistar rats. Values are means ± SE; n = 4 to 6 rats per group. *$P < 0.05$ compared with <20-μm-diameter venules.

critically affect the type of immune response produced. For example, use of *B. pertussis* as an adjuvant favors the production of interleukin-4 and the development of a Th2/IgE-type response.[23] Other reports have shown that Wistar rats can produce significant amounts of specific IgE in response to sensitization with ovalbumin by other procedures,[21,24,25] but additional studies are required to measure changes in specific IgE and IgG under our experimental conditions.

Although IgE and mast cells undoubtedly play a central role in many allergic responses, they may not be the entire story in some species.[26] IgG, which Wistar rats synthesize in significant amounts in response to antigen,[21] has been reported to be a low-affinity ligand for mast cell high-affinity IgE receptors.[27] Several other interstrain differences could influence the magnitude of the late-phase plasma leakage. Endogenous glucocorticoids can affect the amounts of circulating antigen-specific antibody[21] and the magnitude of antigen-induced bronchoconstriction[28] and can reduce allergen-induced plasma leakage.[29] Unanesthetized rats of different strains may exhibit different breathing patterns when challenged,[30] which may, in turn, affect the amount and distribution of aerosolized antigen in the airways. Interstrain differences in the distribution of mast cells and receptors for inflammatory mediators and the rate of removal of extravasated plasma from tissue into the airway lumen[6] or lymphatics[31] could also play a role. Presently, there are few data on interstrain differences to shed light on any of these possibilities.

In this study, our main focus was the mechanism of the early- and late-phase plasma leakage. Thus, we did not measure the amount of airway obstruction or mast cell degranulation after ovalbumin challenge. Such experiments might reveal additional aspects of the model and how it relates to allergic airway responses in humans. Furthermore, analysis of cytokine expression might suggest reasons for the observed differences among rat strains.

Identification of Leaky Vessels in Early- and Late-Phase Leakage

The lectin staining technique allowed us to visualize the vasculature of the entire trachea, and silver nitrate staining and scanning EM allowed us to localize and quantify endothelial gaps. In combination with the extravasation of Monastral blue, these techniques provide evidence that plasma leaks mainly from postcapillary venules in both the early phase and the late phase. The observation that most of the leakage occurred in postcapillary venules whereas the endothelial gaps were more uniformly distributed in venules of different sizes suggests that hemodynamic driving forces play an important role in the leakage. Vessels that leaked in the early phase were similar to those that leak in response to substance P.[10,17] This is not a surprise, as mast-cell-derived mediators, such as histamine and 5-hydroxytryptamine, which are thought to participate in the early phase,[1,2] act on postcapillary venules.[32] The distribution of leaky vessels in the early

Figure 7. Scanning electron micrographs of luminal surface of endothelium of tracheal venules 4 hours after ovalbumin challenge. **A:** Leukocytes are adherent to the endothelium of a collecting venule in a Vibratome section through the entire thickness of tracheal wall (between cartilage rings) with epithelium uppermost. Openings of several smaller venules are also visible in the collecting venule. Smaller vessels (**arrows**) are located just beneath the epithelium (**arrows**). Region in box is enlarged in **B. B:** Migrating leukocytes (**asterisks**) elevate the overlying endothelium. Endothelial gaps (**arrows**) separate from adherent leukocytes. **C:** Oblique slits (**arrows**) and small vertical gaps (**arrowheads**) between endothelial cells of postcapillary venule. **D:** Endothelial gaps (**arrows**) near finger-like cytoplasmic processes but separated from adherent leukocytes and migrating leukocyte (**asterisk**). **E:** Enlargement of endothelial gaps (**arrows**) in **D**. Scale bar, 60 μm (**A**), 10 μm (**B** to **D**), and 2 μm (**E**).

Table 1. Adherent Leukocytes in Rat Tracheal Venules

	Baseline		4 hours after ovalbumin challenge	
	Cells/mm^2	% of total	Cells/mm^2	% of total
Neutrophils	7 ± 2	1.0 ± 0.3%	1437 ± 125*	56.5 ± 2.2%
Eosinophils	1 ± 0.3	0.16 ± 0.03%	80 ± 9*	3.1 ± 0.2%
Lymphocytes	241 ± 57	34.2 ± 4.6%	399 ± 55	15.6 ± 1.6%
Monocytes	302 ± 35	46.9 ± 7.1%	319 ± 47	12.8 ± 2.3%
Unidentified	121 ± 38	17.7 ± 4.6%	299 ± 14*	12.0 ± 1.0%
Total	672 ± 82	100%	2534 ± 159*	100%

Values are expressed as mean ± SE of leukocytes per mm^2 of tracheal surface (n = 4 rats per group; *P < 0.05 compared with baseline). Baseline values are from unsensitized, unchallenged Wistar rats. Challenged values are from sensitized Wistar rats challenged with ovalbumin for 30 minutes and fixed by vascular perfusion 4 hours later. Leukocytes were quantified in tracheal whole mounts after staining by vascular perfusion of biotinylated concanavalin A lectin.

phase corresponded to the distribution of mast cells.[33] In particular, the leakage was greatest in the posterior membrane, where mast cells are most numerous.[33] This observation is consistent with the dependence of early-phase responses on mast cell mediators.[1]

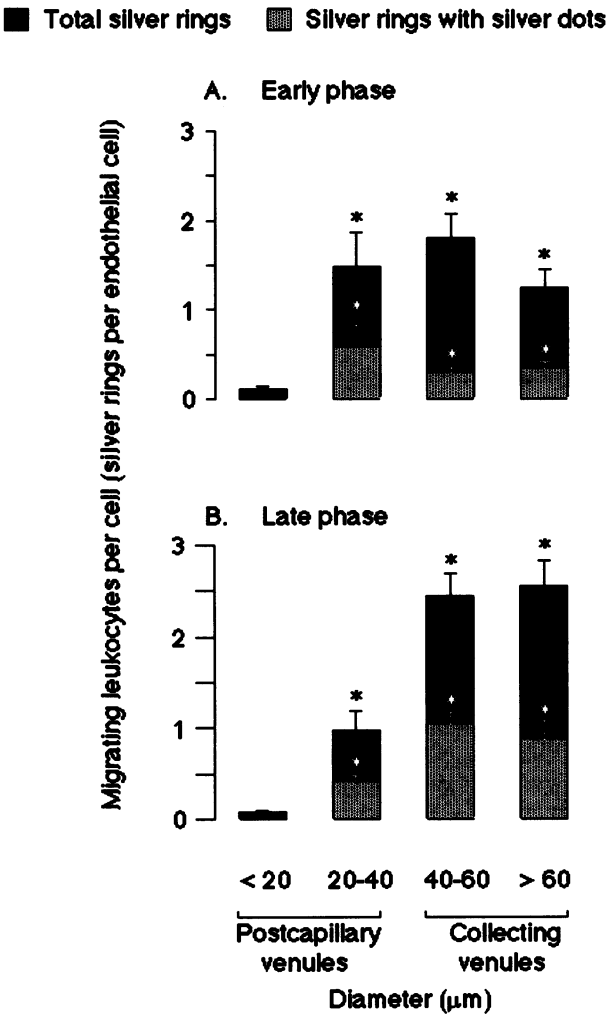


Figure 9. Number of migrating leukocytes, visualized and quantified as silver rings after silver nitrate staining, during early and late phases of plasma leakage after ovalbumin challenge in tracheal mucosa (between cartilage rings) of sensitized Wistar rats. **Black bars**, total silver rings; **gray bars**, silver rings coincident with silver dots. Values are means ± SE; n = 4 to 6 rats per group. *P < 0.05 compared with <20-μm-diameter venules.

The rat trachea is a convenient model for studying the airway microvasculature. Essentially, it is a cylinder that can be cut open and spread as a flat whole mount on a standard microscope slide, so that all blood vessels and leukocytes contained within are visualized in their entirety. The rat trachea is similar in size to human intrapulmonary airways that are thought to be the primary sites of airway obstruction in asthma. The rat extrapulmonary bronchi behave in the same way as the trachea (unpublished observations); future studies might reveal the properties of the smaller intrapulmonary bronchi.

Plasma leakage and the resulting edema can contribute to airway hyperreactivity and obstruction. In the case of massive edema, mechanical forces can cause airway narrowing.[7] Another possible mechanism that requires further investigation is the possibility that plasma leakage can trigger prolonged airway hyperreactivity and bronchoconstriction by the formation and release of blood-derived products such as bradykinin, complement, and fibrin.[6]

The late-phase leakage after antigen exposure lasted approximately 4 hours. In other conditions with prolonged leakage, such as after severe mechanical or thermal trauma, arterioles, capillaries, and venules can all leak.[11] Capillaries in the skin and in skeletal muscle leak selectively in the delayed, prolonged leakage observed after mild thermal or ultraviolet light injury or after exposure to certain bacterial toxins.[11,34] Prolonged irritation can induce plasma leakage from arterioles.[35,36] However, we found no evidence for leakage from capillaries or arterioles in the late phase under our experimental conditions.

Role of Endothelial Gaps in Early- and Late-Phase Plasma Leakage

Our scanning EM observations provided direct evidence for the presence of endothelial gaps in both early- and late-phase leakage. Endothelial gaps found after antigen exposure were similar in appearance and size to those seen after exposure to substance P.[10,17,18] Similarly, the silver dots observed at endothelial cell borders after antigen exposure were indistinguishable from those present after exposure to substance P and have been shown to mark the location of endothelial gaps.[10,17,18] In the re-

gions of the tracheal mucosa between the cartilages, the number of endothelial gaps indicated by silver nitrate staining was not significantly different in the early-phase and late-phase responses. However, in the posterior membrane, the number of gaps in the early phase greatly exceeded that in the late phase. The posterior membrane constitutes approximately 25% of the trachea, but we did not determine how much each of these regions contributed to the overall leakage assessed by Evans blue.

Endothelial gaps present in the early and late phase were indistinguishable when viewed by silver nitrate staining, but some differences were evident by scanning EM. Gaps were more conspicuous in the early phase, because more vertical gaps were present, and the basement membrane was visible through these openings. Similar vertical gaps are present in the initial stage of the response to substance P, when leakage is maximal.[17,18] By comparison, during the late phase, many endothelial gaps resembled the oblique slits that predominate when the response to substance P is subsiding. As the underlying basement membrane was not visible through the oblique gaps, we could not verify whether these structures were open conduits for plasma leakage.

Role of Leukocytes and Other Factors in Late-Phase Leakage

Among the leukocytes that adhered to vasculature in the late phase in Wistar rats, neutrophils were most abundant by far. Although the number of eosinophils increased 80-fold, these cells constituted only 3% of the adherent leukocytes. These observations are in agreement with previous reports of leukocytes in the tissue[5,15,37] and in BAL fluid of Brown Norway rats.[4,5,38,39] In the present study, we focused on adherent or migrating leukocytes because they seemed more likely to affect endothelial permeability than leukocytes that had already migrated into the tissue or airway lumen.

Although late-phase responses in general are believed to be dependent on leukocytes,[1,2] the exact relationship of these cells to microvascular leakage is not clear. To our knowledge, this issue has not been investigated in the respiratory tract, although cell depletion studies suggest that late-phase bronchoconstriction is leukocyte dependent.[2] In antigen-induced plasma leakage in the skin, the early-phase leakage is dependent on mast cells, and the late phase leakage requires both mast cells and neutrophils.[8,40]

Our morphological analysis revealed several features of antigen-induced late-phase plasma leakage in the airways. First, the leakage coincided with regions where endothelial gaps were abundant. Second, most of the leakage occurred upstream of the main sites of leukocyte migration. It has been known for many years that leukocytes can emigrate from blood vessels in skin without an accompanying leakage of particulate tracer.[41] Furthermore, ultrastructural studies of the hamster cheek pouch have shown that neutrophils can pass through the endothelium without any significant escape of plasma.[42]

Additional studies are necessary to determine the significance of the silver dots that coincided with some silver rings at points of leukocyte diapedesis. Although little Monastral blue was found at these sites, we cannot exclude the presence of some plasma leakage.

In addition to creating a potential hole in the endothelium during diapedesis, leukocytes may trigger plasma leakage by releasing inflammatory mediators that induce the formation of endothelial gaps.[3,37] Why plasma leakage and leukocyte migration occur in different regions of the microvasculature is not clear. Differences in endothelial cell phenotype, as evidenced by the heterogeneous distribution of receptors or adhesion molecules, and differences in hydrostatic pressure gradient, blood flow, or shear forces may contribute.

In comparing the present investigation with previous studies of late-phase responses in rats,[15] it appears that the peak of late-phase plasma leakage at 4 to 6 hours coincides with or precedes the peak of airway responsiveness, which is typically at 8 hours.[43] The peak of late-phase leakage also occurs near the peak of neutrophil influx into the tissue or airway lumen[4,5,39] but precedes the peak of eosinophil influx at 72 hours.[5,39] This timing is consistent with other evidence from depletion studies that neutrophils participate in late-phase plasma leakage[8] but that eosinophils are probably too few and too late to have a major effect on leakage.

Conclusions

We conclude that the antigen challenge induces early and late phases of plasma leakage in the respiratory tract of rats. In both phases, postcapillary venules are the predominant source of leakage. Endothelial gaps are abundant in the leaky vessels, but most of the leukocyte adhesion and migration occurs downstream in collecting venules. Endothelial gaps that form in response to antigen resemble the gaps that form after exposure to histamine, serotonin, or substance P.

Acknowledgments

We thank the Department of Pathology at UCSF for use of their scanning electron microscope and Dr. Marc Dupuis for helpful discussions.

References

1. O'Byrne P, Dolovich J, Hargreave FE: Late asthmatic responses. Am Rev Respir Dis 1987, 136:740–751
2. Lemanske RF, Kaliner MA: Late phase allergic reactions. Allergy Principles and Practice, vol 1, ed 4. Edited by Middleton E. St. Louis, Mosby, 1993, pp 320–361
3. Walls AF, Rhee K, Gould DJ, Walters C, Robinson C, Church MK, Holgate ST: Inflammatory mediators and cellular infiltration of the lungs in a guinea pig model of the late asthmatic reaction. Lung 1991, 169:227–240
4. Renzi PM, Olivenstein R, Martin JG: Inflammatory cell populations in the airways and parenchyma after antigen challenge in the rat. Am Rev Respir Dis 1993, 147:967–974
5. Schneider T, van Velzen D, Moqbel R, Issekutz AC: Kinetics and

quantitation of eosinophil and neutrophil recruitment to allergic lung inflammation in a brown Norway rat model. Am J Respir Cell Mol Biol 1997, 17:702–712
6. Persson CG: Plasma exudation in the airways: mechanisms and function. Eur Respir J 1991, 4:1268–1274
7. Brown RH, Zerhouni EA, Mitzner W: Airway edema potentiates airway reactivity. J Appl Physiol 1995, 79:1242–1248
8. Sekiya S, Yamashita T, Sendo F: Suppression of late phase enhanced vascular permeability in rats by selective depletion of neutrophils with a monoclonal antibody. J Leukocyte Biol 1990, 48:258–265
9. Majno G, Palade GE: Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. J Biophys Biochem Cytol 1961, 11:571–605
10. McDonald DM: Endothelial gaps and permeability of venules in rat tracheas exposed to inflammatory stimuli. Am J Physiol 1994, 266: L61–L83
11. Majno G: The Maude Abbott Lecture, 1991. The capillary then and now: an overview of capillary pathology. Mod Pathol 1992, 5:9–22
12. Neal CR, Michel CC: Transcellular gaps in microvascular walls of frog and rat when permeability is increased by perfusion with the ionophore A23187. J Physiol 1995, 488:427–437
13. Dvorak AM, Kohn S, Morgan ES, Fox P, Nagy JA, Dvorak HF: The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. J Leukocyte Biol 1996, 59:100–115
14. Simionescu N: Cellular aspects of transcapillary exchange. Physiol Rev 1983, 63:1536–1579
15. Bolton PB, Lefevre P, McDonald DM: Salmeterol reduces early and late phase plasma leakage and leukocyte adhesion in rat airways. Am J Respir Crit Care Med 1997, 155:1428–1435
16. Thurston G, Baluk P, Hirata A, McDonald DM: Permeability related changes revealed at endothelial cell borders in inflamed vessels by lectin staining. Am J Physiol 1996, 271:H2547–H2562
17. Baluk P, Hirata A, Thurston G, Fujiwara T, Neal CR, Michel CC, McDonald DM: Endothelial gaps: time course of formation and closure in inflamed venules. Am J Physiol 1997, 272:L155–L170
18. Hirata A, Baluk P, Fujiwara T, McDonald DM: Location of focal silver staining at endothelial gaps in inflamed venules examined by scanning electron microscopy. Am J Physiol 1995, 269:L403–L418
19. Pauwels R, Bazin H, Platteau B, Van der Stratten M: The influence of antigen dose on IgE production in different rat strains. Immunology 1979, 36:151–157
20. Smith SR, Petillo J: IgE production in five inbred rat strains following immunization with alum-precipitated egg albumin. Int Arch Allergy Appl Immunol 1976, 52:21–31
21. Peers SH, Duncan GS, Flower RJ: Development of specific antibody and in vivo response to antigen in different rat strains: effect of dexamethasone and importance of endogenous corticosteroids. Agents Actions 1993, 39:174–181
22. Chiba Y, Misawa M: Strain differences in change in airway responsiveness after repeated antigenic challenge in 3 strains of rats. Gen Pharmacol 1993, 24:1265–1272
23. Mu H-H, Sewell WA: Enhancement of interleukin-4 production by pertusssis toxin. Infect Immun 1993, 61:2834–2840
24. Vianna EO, Garcia-Leme J: Allergen-induced airway inflammation in rats: role of insulin. Am J Respir Crit Care Med 1995, 151:809–814
25. Lima MC, Prouvost-Danon A, de Silva PM, Chagas MS, Calheiros AS, Cordeiro RS, Latine D, Bazin H, Ryan US, Martins MA: Studies on the mechanisms involved in antigen-evoked pleural inflammation in rats: contribution of IgE and complement. J Leukocyte Biol 1997, 61:286–292
26. Galli SJ: Complexity and redundancy in the pathogenesis of asthma: reassessing the roles of mast cells and T cells. J Exp Med 1997, 186:343–347
27. Benhamou M, Berenstein EH, Jouvin MH, Siraganian RP: The receptor with high affinity for IgE on rat mast cells is a functional receptor for rat IgG2a. Mol Immunol 1994, 31:1089–1097
28. Turner DJ, Myron P, Powell WS, Martin JG: The role of endogenous corticosterone in the late-phase response to allergen challenge in the Brown Norway rat. Am J Respir Crit Care Med 1996, 153:545–550
29. Nilsson MC, Aberg P, Erjefält I, Persson CGA: Effect of insufflated budesonide powder on allergen challenge-induced late phase inflammatory exudation in rat large airways. Am J Respir Crit Care Med 1997, 155:A879
30. Piechuta H, Smith ME, Share NN, Holme G: The respiratory response to sensitized rats to challenge with antigen aerosols. Immunology 1979, 38:385–392
31. Erjefält I, Luts A, Persson CGA: Appearance of airway absorption and exudation tracers in guinea pig tracheobronchial lymph nodes. J Appl Physiol 1993, 74:817–824
32. Majno G, Palade G, Schoefl GI: Studies on inflammation. II. The site of action of histamine and serotonin in vascular tree: a topographic study. J Biophys Biochem Cytol 1961, 11:607–630
33. Tam EK, Calonico LD, Nadel JA, McDonald DM: Globule leukocytes and mast cells in the rat trachea: their number, distribution, and response to compound 48/80 and dexamethasone. Anat Embryol 1988, 178:107–118
34. Joris I, Cuénoud HF, Doern GV, Underwood JM, Majno G: Capillary leakage in inflammation: a study by vascular labeling. Am J Pathol 1990, 137:1353–1363
35. Cuénoud HF, Joris I, Langer RS, Majno G: Focal arteriolar insudation: a response of arterioles to chronic nonspecific irritation. Am J Pathol 1987, 127:592–604
36. Pietra GG, Johns LW: Confocal- and electron-microscopic localization of FITC-albumin in H_2O_2-induced pulmonary edema. J Appl Physiol 1996, 80:182–190
37. Sapienza S, Eidelman DH, Renzi PM, Martin JG: Role of leukotriene D4 in the early and late pulmonary responses of rats to allergen challenge. Am Rev Respir Dis 1990, 142:353–358
38. Elwood W, Barnes PJ, Chung KF: Airway hyperresponsiveness is associated with inflammatory cell infiltration in allergic Brown Norway rats. Int Arch Allergy Immunol 1992, 99:91–97
39. Chapman ID, Lee AJ, Thompson DC, Templeton AGB, Milne AAY: Time course of allergen-induced leucocyte accumulation in actively sensitized Brown Norway rats. Am J Respir Crit Care Med 1996, 153:A219
40. Mekori YA, Galli SJ: [^{125}]Fibrin deposition occurs at both early and late intervals of IgE-dependent or contact sensitivity reactions elicited in mouse skin: mast cell-dependent augmentation of fibrin deposition at early intervals in combined IgE-dependent and contact sensitivity reactions. J Immunol 1990, 145:3719–3727
41. Hurley JV: Acute inflammation: the effect of concurrent leucocytic emigration and increased permeability on particle retention by the vascular wall. Br J Exp Pathol 1964, 45:627–633
42. Lewis RE, Granger HJ: Diapedesis and the permeability of venous microvessels to protein macromolecules: the impact of leukotriene B_4 (LTB_4). Microvasc Res 1988, 35:27–47
43. Xu LJ, Eidelman DH, Bates JHT, Martin JG: Late response of the upper airway of the rat to inhaled antigen. J Appl Physiol 1990, 69:1360–1365